INFECTION AND IMMUNITY, May 1988, p. 1382–1384
0019-9567/88/051382-03$02.00/0

Vol. 56, No. 5

NOTES

Mitogenic Activities of Synthetic *Escherichia coli* Lipid A and a Synthetic Partial Structure (Tripalmitoyl Pentapeptide) of *E. coli* Lipoprotein

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Received 30 November 1987/Accepted 21 January 1988

Synthetic *Escherichia coli* lipid A and synthetic *S*-[2,3-bis-(palmitoyloxy)propyl]-*N*-palmitoylpentapeptide (tripalmitoyl pentapeptide [TPP]), representing the mitogenically active principles of bacterial lipopolysaccharide (LPS) and lipoprotein, respectively, were compared for their mitogenic activities on splenocytes of LPS responder (BALB/c) and LPS-low-responder (C3H/HeJ) mice. Whereas lipid A was active only in LPS-responder mice, TPP resulted in mitogenic activation of B lymphocytes from both LPS-responder and LPS-low-responder mice. When the mitogens were added simultaneously, mainly additive effects of both activators were observed. The data suggest that two different B-lymphocyte populations are responding to these two mitogens.

Lipopolysaccharides (LPS) are common constituents of the gram-negative bacterial cell wall (10). They represent the endotoxins of gram-negative bacteria, exhibiting numerous biological activities in higher organisms; mitogenic and polyclonal activation of murine B lymphocytes is well documented (2). The mitogenic principle, like that of many other biological parameters, is located in the lipid A moiety of the LPS molecule (5). For endotoxic activities of LPS in higher organisms, a functionally intact LPS response gene is required since the LPS-low-responder (LPS-LR) mouse strain C3H/HeJ does not respond to lipid A as a mitogen (11). However, it has been proposed that a biosynthetic precursor of lipid A, named precursor Ia and containing only 2 mol equivalents of ester-linked fatty acids compared with 4 mol equivalents in lipid A (6), is mitogenically active in both LPS-responder (LPS-R) and LPS-LR mice (12). By using synthetic lipid A (7) and precursor Ia (8), it could be demonstrated that an intact LPS response gene is also required for a mitogenic response to precursor Ia (4). Thus, the reported activity of natural precursor Ia in LPS-LR mice may be due to a contamination of precursor Ia with other cell wall components that copurified during its preparation from bacteria.

The lipoprotein of gram-negative bacteria is also known as a potent mitogen for B lymphocytes; it is equally active in LPS-R and LPS-LR mice (9). Since it is known that even highly purified preparations of endotoxins contain trace amounts of protein (less than 0.02%) independent of the extraction procedure, we asked the question whether small amounts of lipoprotein, which alone would not support B-lymphocyte activation, could act on the activation by LPS or lipid A synergistically. The mitogenic principle of lipoprotein constitutes a tripalmitoyl pentapeptide (TPP), which also has been chemically synthesized (13) and which has been shown to be a potent mitogen and polyclonal activator for B lymphocytes of both LPS-R and LPS-LR mice (2). Therefore, we designed experiments to determine whether and how TPP and lipid A, alone or in combination, act on B lymphocytes of LPS-R and LPS-LR mice.

Synthetic *Escherichia coli* lipid A was kindly provided by

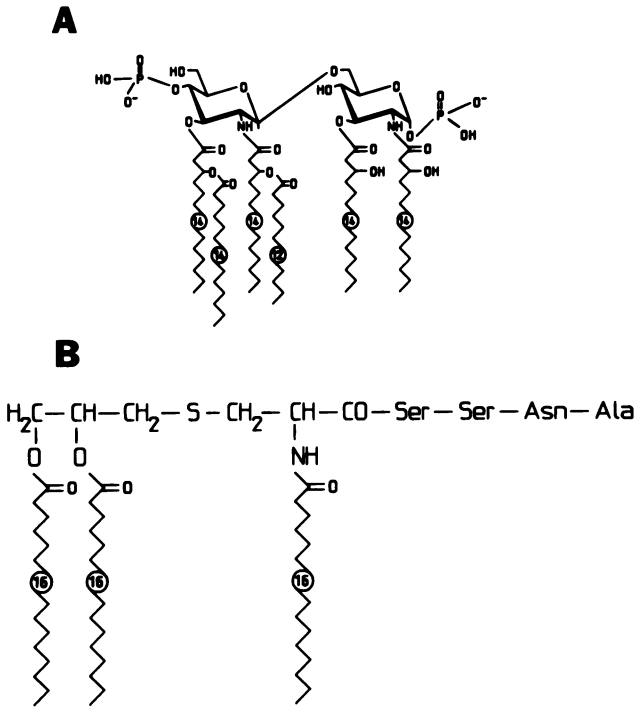

FIG. 1. Chemical structures of synthetic *E. coli* lipid A (A) and synthetic TPP (B).

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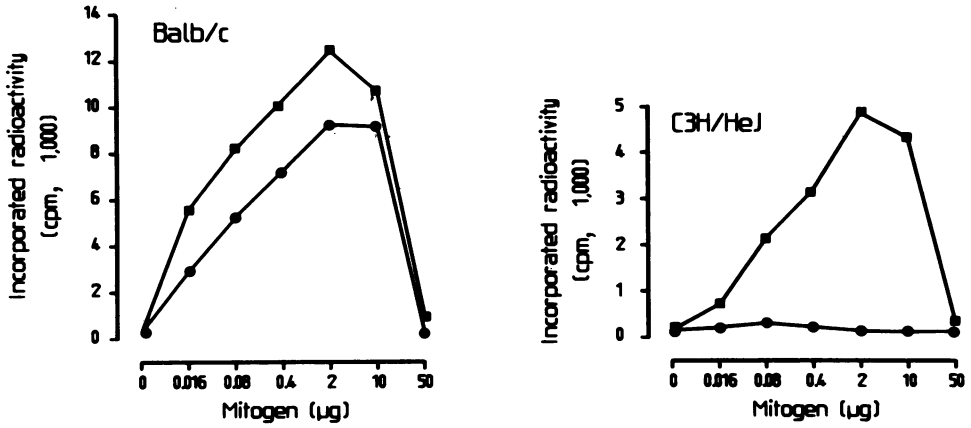

FIG. 2. Mitogenicity of synthetic *E. coli* lipid A (●) and synthetic TPP (■) for splenocytes of LPS-R (BALB/c) and LPS-LR (C3H/HeJ) mice. Mitogenicity was determined by cultivating 8×10^5 spleen cells in 200 μl of Iscove medium for 48 h in the presence of mitogen followed by [^{3}H]thymidine incorporation for another 16 h.

S. Kusumoto (Osaka, Japan), and synthetic TPP was a gift of K. H. Wiesmüller (Tübingen, Federal Republic of Germany). The chemical structures of these compounds are shown in Fig. 1. BALB/c mice (LPS-R) were obtained from the Lippische Versuchstieranstalt (Hannover, Federal Republic of Germany), and C3H/HeJ mice were obtained from the Max-Planck Institut für Immunbiologie (Freiburg, Federal Republic of Germany). Mitogenicity was determined in spleen cells by [^{3}H]thymidine incorporation as described in detail elsewhere (4). Serum-free conditions were used to avoid interactions of the mitogens with serum constituents such as complement components, high-density lipoprotein, or naturally occurring antibodies. Briefly, 8×10^5 spleen cells in 200 μl of Iscove incomplete medium were preincubated with graded amounts of lipid A or TPP for 48 h, followed by the addition of [^{3}H]thymidine and further incubation for 16 h. All experiments were done twice in duplicate. Figure 2 shows the mitogenic activity of lipid A and TPP for lymphocytes of LPS-R (BALB/c) and LPS-LR (C3H/HeJ) mice. Lipid A was only active in LPS-R mice, yielding a maximal stimulation with 2 and 10 μg. TPP was active in both mouse strains, exhibiting an optimal response with 2 μg.

In a second set of experiments, the two polyclonal activators were added simultaneously to the splenocytes; the concentration of one mitogen was kept constant, and that of the second was varied (Table 1). With higher doses of the two activators, the radioactivity incorporated reflected an additive rather than a synergistic effect of the two mitogens. Interestingly, at the highest lipid A concentration used (10 μg), inhibition of the TPP response was observed in LPS-LR lymphocytes (Table 1), which cannot be explained at present. Figure 3 illustrates the results obtained when the optimal concentration of one of both activators was kept constant (2 μg) and the other was added in graded amounts. Essentially the same results were obtained when synthetic precursor Ia instead of lipid A was used or when the two activators were first mixed, lyophilyzed, and resolved to ascertain a homogenous mixture (data not shown).

Our results show that lipid A and TPP, the principal mitogens of bacterial LPS and lipoprotein, respectively, do not act synergistically on each other with regard to their mitogenic activity in LPS-R and LPS-LR mice. The precursor Ia preparation used by Vogel et al. contained 0.23% of protein and was mitogenically active with 1 μg (12). Even if all protein determined would be lipoprotein, the amount

TABLE 1. Mitogenic activity of synthetic *E. coli* lipid A (compound 506) and TPP added simultaneously to lymphocytes of LPS-R (BALB/c) and LPS-LR (C3H/HeJ) mice

Mouse strain	Amt of lipid A (μg)	Incorporated [^{3}H]thymidine (cpm) when the following amts (μg) of TPP were added:					
		0	0.016	0.08	0.4	2	10
BALB/c	0	302	5,583	8,201	9,934	12,460	10,710
	0.016	2,923	5,120	6,770	10,134	12,937	13,048
	0.08	5,232	5,729	6,628	11,022	13,318	14,518
	0.4	6,913	7,204	8,491	12,788	15,675	18,431
	2	9,266	9,070	10,056	14,084	19,103	21,718
	10	9,171	11,616	12,788	15,213	17,396	19,226
C3H/HeJ	0	95	730	2,176	3,182	4,848	4,313
	0.016	93	732	1,811	3,255	4,705	5,688
	0.08	92	825	2,034	3,365	4,931	5,736
	0.4	87	784	1,988	3,754	4,867	5,631
	2	73	1,024	2,024	3,566	3,975	3,739
	10	75	643	889	1,232	1,590	1,632

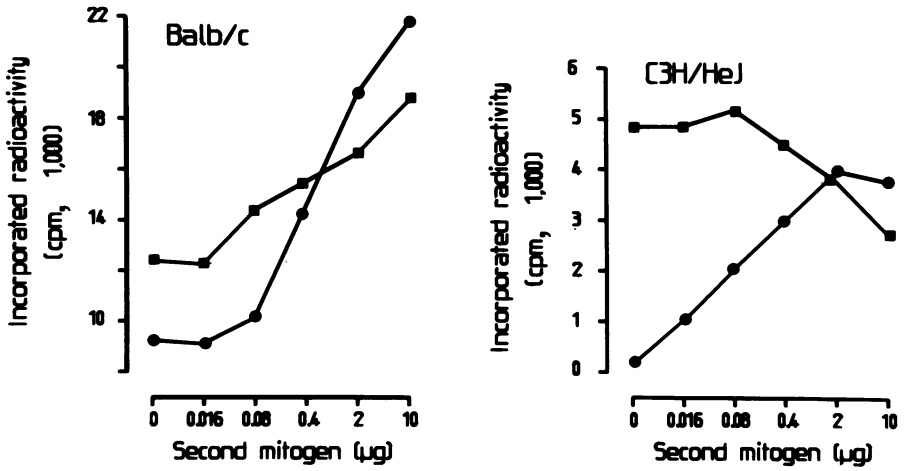

FIG. 3. Mitogenicity of simultaneously added synthetic *E. coli* lipid A and synthetic TPP for splenocytes of LPS-R (BALB/c) and LPS-LR (C3H/HeJ) mice. Symbols: (●) 2 μg of lipid A was added together with graded amounts of TPP; (■) 2 μg of TPP was added together with graded amounts of lipid A. See also the legend to Fig. 2.

would not exceed 2.3 ng. Therefore, it seems unlikely that the assumed contaminating cell wall component, responsible for the mitogenic activity of natural precursor Ia in LPS-LR mice, is bacterial lipoprotein. Assuming that polyclonal activation initiates the maximal response of each individual cell (1), the data suggest that two different B-cell populations are responding to these two mitogens.

We thank S. Kusumoto (Osaka, Japan) and K. H. Wiesmüller (Tübingen, Federal Republic of Germany) for synthetic lipid A and synthetic TPP, respectively. The expert technical assistance of U. Albert is gratefully acknowledged.

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